The Promise of Third-Generation Recombinant Therapy and Gene Therapy

Catherine S. Manno

Recombinant factor VIII and IX products have well-established efficacy and safety records. However, concerns about the possibility of viral transmission have prompted efforts to develop recombinant products that are free of added human and animal proteins. The currently licensed second-generation recombinant factor VIII concentrates were introduced in 2000. Two new third-generation products, manufactured without any human- or animal-derived materials, are currently in development and clinical testing. As an alternative to exogenous factor replacement, gene therapy is under investigation for use in the treatment of hemophilia. Gene therapy involves the stable insertion of a functional gene for long-term expression and secretion of endogenous factor VIII or IX protein. Methods used to date have been based on retroviral, adenoviral, and adeno-associated viral vectors, as well as nonviral electroporation. Three phase I trials using these approaches have been completed as of 2002, and one more is ongoing. This article reviews the results of recent clinical studies investigating third-generation recombinant products and gene-based approaches to hemophilia treatment.

Semin Hematol 40(suppl 3):23-28.

FOR THE PAST 10 years, recombinant factor concentrates have been prescribed for the treatment and prevention of bleeding episodes in patients with hemophilia. The safety record of these products is superb; to date, no confirmed cases of viral transmission have been linked to their use. Any remaining perception that viral disease may occur through infusion of factor concentrates stems from the tragic infection of the majority of adult patients with severe hemophilia who were given contaminated concentrates two decades ago.

Despite this improved safety, concerns persist regarding the necessary addition of human albumin to current first-generation recombinant factor VIII concentrates during the production process. The theoretical concern that albumin might transmit a known or as yet unrecognized infectious disease has prompted the Medical and Scientific Advisory Council (MASAC) of the National Hemophilia Foundation to recommend that efforts be made to remove human albumin from recombinant factor VIII products. Moreover, MASAC recommends that all human and bovine proteins be eliminated from the manufacturing process of recombinant products.[1]

One second-generation recombinant factor VIII concentrate, first introduced in 2000 by Bayer, contains reduced human albumin in the final formulation compared to first-generation products. Another, ReFacto, manufactured by Wyeth, is derived from a B-domain–deleted F.VIII construct. Third-generation products that are prepared without any added human- or animal-derived materials, are currently in development.

Because of the theoretical safety risks associated with the use of recombinant products and practical problems associated with intravenous infusion of factor concentrates, gene therapy is currently being investigated as a potential treatment for hemophilia. The goal of gene therapy is to provide stable insertion and long-term expression of the gene for either factor VIII or IX. Clinical trials of gene therapy for hemophilia were initiated in the United States in 1999, generating much interest and, more recently, preliminary results.

This review assesses the potential benefits and indications for third-generation recombinant products, as well as the current potential for gene therapy, in the treatment of hemophilia. Clinical studies testing the efficacy of these strategies are reviewed, and preliminary results, where available, are presented.

First- and Second-Generation Recombinant Products

Ten years ago, recombinant factor concentrates were licensed for the treatment of hemophilia A in the United States. In 1992, the first recombinant factor VIII product (Recombinate, Baxter Healthcare Corp, Glendale, CA) emerged, followed in 1993 by a second factor VIII product (Kogenate, Bayer Corp, Elkhart, IN). Two years later a recombinant factor IX product

From the Department of Pediatrics, University of Pennsylvania School of Medicine, and the Division of Hematology, The Children's Hospital of Philadelphia, Philadelphia, PA.

Address correspondence to Catherine S. Manno, MD, The Children's Hospital of Philadelphia, 34th St and Civic Center Blvd, Philadelphia, PA 19104.

0037-1963/03/4003-3005$30.00/0

doi:10.1016/S0037-1963(03)00180-X

(BeneFix, Genetics Institute Inc, Cambridge, MA) appeared, followed in 1999 by a third factor VIII product (ReFacto, Genetics Institute Inc).

The factor VIII products currently available in the United States include Recombinate, Kogenate FS (Bayer Corp), ReFacto, and Helixate FS (Bayer Corp). All of these products use human albumin at some point in the production process. Albumin, although derived from pools of plasma, has an excellent safety record. A recent report found no confirmed reports of viral transmission from the use of human albumin in the United States in 53 years.[2] Of the recombinant concentrates used to date, no confirmed case of disease transmission has been associated with these products.

The recombinant products reconstitute to about 2.5 mL to 10 mL of volume and, except for ReFacto, represent the full-length factor VIII molecule. ReFacto is B-domain–deleted factor VIII. Of the recombinant factor VIII products, Recombinate is the only product that uses albumin as a stabilizer in the product's final formulation. The other recombinant factor VIII products use a cell culture medium containing human plasma protein solution and recombinant human insulin. The FS products, Kogenate FS and Helixate FS, are formulated with sucrose instead of albumin as a stabilizer. Of the recombinant concentrates used to date, no confirmed case of disease transmission has been attributed to these products.

Despite the proven safety record and continued improvements in the manufacturing process of recombinant products, concern persists about the potential for viral transmission with infusion of any product that contains plasma-derived proteins. Although the well recognized transfusion-transmitted viruses including HIV, HBV, and HCV are readily inactivated by heat or solvent-detergent treatment, the risk of transmission of other, not yet recognized, infectious agents that resist current inactivation techniques still exists.

Third-Generation Factor VIII Products

The two third-generation recombinant factor VIII concentrates, produced without exogenous animal or human protein are in development, including a B-domain–deleted recombinant factor VIII (BDDrFVIII) from Wyeth (Philadelphia, PA), and a product from Baxter Healthcare Corporation that is made using a full-length factor VIII concentrate, made with a protein-free method (rAHF-PFM). Neither of these products is licensed.

For the BDDrFVIII product, several changes were made to the manufacturing process to accommodate production in a serum- and albumin-free medium. All these changes were subject to the constraint that they would not affect product quality or purity.

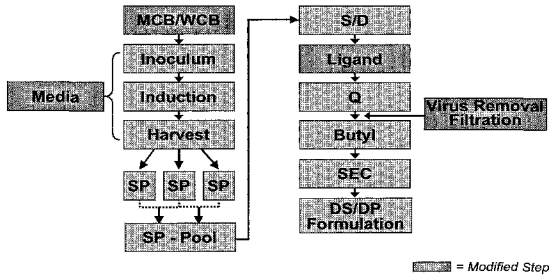

Figure 1. Manufacturing process of the third-generation BDDrFVIII product. The dark boxes indicate modified steps—the master cell bank/working cell bank (MCB/WCB), media, and addition of ligand separation and viral filtration steps—that distinguish this product from that of previous BDDrFVIII products. SP, sulfopropyl; S/D, solvent/detergent; Q, quaternary aminomethyl; SEC, size exclusion chromatography; DS/DP, drug substance/drug product.

The manufacture of the new BDDrFVIII required modification of several steps that distinguish it from the earlier-generation factor VIII products (Fig 1). The master cell bank and working cell bank are in a medium containing recombinant insulin as the only added protein. The cells are derived from the B-domain–deleted factor VIII master cell lineage and grown in an albumin-free, serum-free medium.

A novel chemically synthesized ligand for extraneous protein removal replaces the monoclonal antibody used in the earlier production process. The ligand also removes equivalent Chinese hamster ovary protein, resulting in a product with purity comparable to that seen in previous recombinant factor VIII concentrates (>99% clotting protein).

The addition of a viral filtration step is new for the manufacturing of recombinant factor VIII products. This step differs from chromatography and solvent-detergent inactivation steps in that its size-selective mechanisms removes medium- and large-sized viruses completely. The small-sized B-domain–deleted factor VIII is able to pass through the small pores (35 nm) of the filter.

Studies have validated the effectiveness of the BDDrFVIII manufacturing process, which combines solvent-detergent step, viral filtration, and affinity chromatography steps in reducing viral load. Trials with this product have not yet been initiated.

The rAHF-PFM product is derived from production cell lines similar to those of its earlier product, Recombinate. The rAHF-PFM product has a primary structure identical to that of Baxter's previous recombinant antihemophilic factor. This concentrate will have a reconstituted volume of 5 mL, smaller than that of Recombinate. No human or animal materials are used in the cell culture process, purification process, or for stabilization of the final formulation. A dedicated viral inactivation step employs solvent-detergent treatment. The new product retains the

Table 1. Viral and Nonviral Strategies Used in Gene-Based Therapies for Hemophilia

Viral vectors	
Retrovirus	Requires gene integration for long-term expression
Adenovirus	Large capacity for transgene expression; generates strong immune response
Adeno-associated virus	Parvovirus associated with no human disease, small coding region
Nonviral method	
Electroporation	Electric impulses used to open pores in the cell membrane to facilitate DNA integration either ex vivo or in vivo

physicochemical characteristics of Recombinate and is predicted to have comparable levels of safety, tolerability, and hemostatic efficacy.

Safety and efficacy studies for rAHF-PFM have been completed in rat and mouse models. In addition, seven clinical trials are being planned or conducted. Ongoing studies include a pivotal phase III trial with 111 previously treated adult patients, a continuation study with 80 previously treated patients, a surgical study with 48 patients, and a study of previously treated children. Planned studies include safety and efficacy in previously untreated patients, a postlicensure phase IV study, and a clinical study in Japan (unpublished data, Baxter BioScience, 2002).

The pivotal phase III trial, a randomized, double-blind, crossover study involving 111 previously treated patients, compared the pharmacokinetics and efficacy of rAHF-PFM with those of Recombinate in 108 patients over 10 years of age. The two products were shown to be bioequivalent. Pharmacokinetic parameters and hemostatic efficacy were similar between the two products as well. No serious adverse events and an acceptable incidence of drug-related adverse events were reported with the use of rAHF-PFM (unpublished data, Baxter BioScience, 2002).

Gene Therapy Clinical Trials

To date, three gene transfer studies in patients with hemophilia have been completed, and currently one trial is enrolling subjects. These studies are evaluating several approaches to gene transfer utilizing viral vectors and nonviral methods (Table 1).

Studies of retroviral, adenoviral, or adeno-associated viral (AAV) vectors have been completed or are enrolling subjects. Retroviral vectors require integration into dividing cells for long-term expression. Adenoviral vectors have the advantage of a large capacity to accommodate a transgene. The adenoviral-based vectors cause an immune response in recipients that may contribute to short-lived transgene expression. AAV is a small parvovirus that does not cause human disease, but its small coating region does not accommodate large genes, such as the full-length factor VIII gene.

One method for transferring genes to cells is electroporation. Electroporation requires brief application of electric impulses to cells, either in vivo or ex vivo, which creates transient pores in the membrane and allows the polar and/or charged agents (such as DNA, RNA, some dyes) to enter the cell. In ex vivo electroporation, autologous cells into which a gene has been inserted can be characterized before reimplantation into the patient to ensure the gene of interest is being expressed at a particular level.

All trials of gene therapy in hemophilia must assess safety issues of the approach. Risks being assessed include the possibilities of inhibitor formation factor VIII or factor IX, insertional mutagenesis, and germline transmission (ie, presence of transgene in semen with potential for transmission to offspring). Germline transmission is not a concern with an ex vivo approach, as it is with some virally based approaches, since the transgene is applied to specific cells and not administered parenterally. The goal of gene transfer therapy is to achieve sustained transgene expression; expression resulting in factor VIII or IX levels as modest as 1% to 3% would likely benefit patients with hemophilia. Maintaining factor levels above 1% is key to the success of prophylaxis regimens and the reason patients with mild or moderate disease have fewer bleeding complications. A successful gene therapy approach in early trials would be defined as the conversion of a patient with severe disease into one with mild or moderate disease. In addition, overexpression of the transgene is not likely to harm patients, because the upper limit of the normal range is 150% for factor VIII or IX.

Completed Gene Therapy Trials

Gene therapy trials which have been completed include intravenous (IV) infusion of a retroviral vector expressing a B-domain–deleted factor VIII (sponsor, Chiron Corp), the ex vivo transfection of autologous fibroblasts with a plasmid encoding B-domain–deleted factor VIII (sponsor, Transkaryotic Therapies, Inc), and intramuscular injection of recombinant AAV-expressing human factor IX (sponsor, Avigen, Inc).

The mechanism of retroviral transduction is well understood. The retroviral RNA is altered so that genes that facilitate viral replication are removed, and genes necessary for reverse transcription, factor VIII, and other supporting genes are kept or inserted. The virus infects hepatic cells, and transcription of the plasmid factor VIII RNA to DNA occurs. The factor

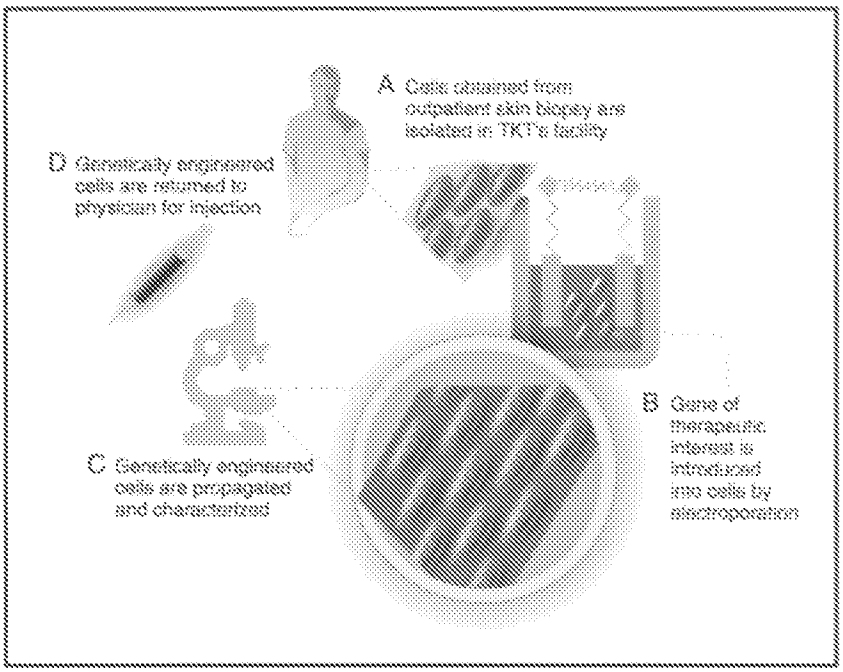

Figure 2. Procedure for the ex vivo transfection of factor VIII into autologous human fibroblasts. Cells obtained by skin biopsy are isolated and cloned, followed by electroporation to induce introduction of a plasmid encoding B-domain–deleted factor VIII into the cellular DNA. Cells are then propagated and ultimately reinjected into the omentum. Figure courtesy of Transkaryotic Therapies, Inc, Cambridge MA; used with permission.

VIII gene is expressed, and the protein is produced and secreted.

The clinical trial involving the retroviral vector was an open-label dose-escalation study. Doses were infused intravenously and ranged from 1×10^8 colony-forming units (cfu)/kg up to 8×10^8 cfu/kg. Fourteen subjects were enrolled and are currently in follow-up. Preliminary results have shown occasional measurements of factor VIII expression above 1%, but no persistent transgene expression, and no serious adverse effects related to the vector.[3]

In the TKT gene therapy trial studying ex vivo transfection of autologous fibroblasts with a plasmid-encoding BDDFVIII, six patients with severe hemophilia A were enrolled initially. Subjects underwent skin biopsy to obtain fibroblasts. The gene for factor VIII was electroporated into the fibroblasts ex vivo. The fibroblasts identified as producing factor VIII were isolated, cloned, and implanted into the patients' omentums (Fig 2).

The initial results from the first six patients enrolled in this trial were published by Roth and colleagues in June 2001.[4] In addition to demonstrating that implantation of the genetically altered fibroblasts was safe, the results showed that four of the six patients had factor VIII activity that rose above previous baseline levels; these patients also had a decreased incidence of bleeding episodes and decreased use of factor VIII concentrate. In one patient increased factor VIII levels persisted for up to 10 months following the procedure before falling back below 1%. Despite initial promise, long-term expression has not been shown. Efficacy results for the total of 12 subjects enrolled in this trial are yet to be published.

The trial of AAV-mediated, muscle-directed, factor IX gene therapy employed an open-label, dose-escalation design with low-, medium-, and high-dose cohorts. Eight subjects were enrolled, three each in the low- and medium-dose cohorts and two in the high-dose cohort. Subjects received intramuscular injection of the vector genomes (vg) in 2.0×10^{11} vg/kg, 6.0×10^{11} vg/kg, and 1.8×10^{12} vg/kg, respectively. Subjects were assessed for plasma factor IX levels, muscle enzymes, and the development of inhibitors.

At 2 and 6 months after injection of the vector into muscle, subjects underwent a muscle biopsy to assess the efficacy of gene transfer. For all of the subjects receiving the vector, adverse events were minimal.

Muscle tissue obtained at biopsy was analyzed by Southern blot analysis, immunofluorescence assay, and immunohistochemistry. Southern blot analysis of genomic DNA showed the presence of vector-specific sequences in five of nine specimens. Factor IX expression was demonstrated in the myocytes of all patients by immunofluorescent or immunohistochemical staining. Despite evidence of transgene insertion, only two of eight patients demonstrated expression of factor IX above 1%. Evidence of a dose response was not seen. Overall, the vector was well tolerated, with no significant systemic or local toxicities observed in the subjects enrolled.[5,6]

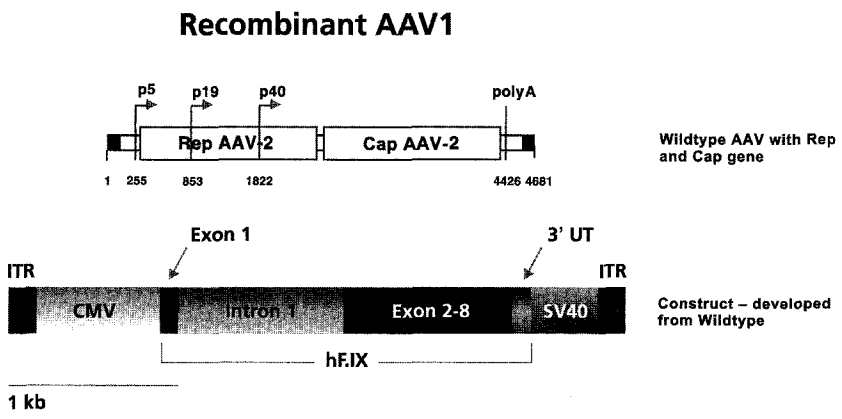

Figure 3. Construct derived from wild-type AAV vector used in the Avigen, Inc, study of muscle-directed factor IX gene therapy. The vector includes portions of factor IX complementary DNA (cDNA) and uses a nonspecific cytomegalovirus enhancer/promoter. Reprinted with permission from Dr. Katherine High.

Other Gene Therapy Trials

Two other hemophilia gene therapy trials deserve mention, the intravenous infusion of an adenoviral vector expressing factor VIII (sponsor, GenStar Therapeutics) and the intrahepatic arterial infusion of an AAV vector expressing factor IX (sponsor, Avigen, Inc).

The former trial was a multicenter phase I study using escalating intravenous doses of a construct called Max-Ad (GenStar Therapeutics, San Diego, CA), a gutted adenoviral-based vector that directs the full-length factor VIII cDNA to somatic cells. The rationale for a gutless vector is that eliminating most viral coding sequences will minimize immune toxicity.[7] Enrollment was of subjects with hemophilia who may be human immunodeficiency virus (HIV)-positive ($CD4^+$ count > 300 cells/μL) and hepatitis C virus (HCV)-positive but negative for HCV by polymerase chain reaction (PCR) assay.

One male subject was treated in this trial. During the first 24 hours after infusion he developed thrombocytopenia and a prolonged prothrombin time. Despite an increase in measured plasma factor VIII levels up to 3%, he suffered a joint bleed requiring factor VIII treatment. After 4 days, his platelet count and coagulation parameters had normalized.[8] This trial has been discontinued due to concerns of toxicity and slow subject accrual.

Finally, there is the phase I dose-escalation study involving intrahepatic arterial injection of an AAV-human factor IX construct with a liver-specific promoter (Coagulin B, Avigen, Inc, Alameda, CA). The wild-type AAV construct is similar to the construct used for the muscle-directed, factor IX gene therapy study, but this vector incorporates a liver-specific promoter in place of the cytomegalovirus enhancer/promoter (Fig 3).

Miao et al reported that hepatocytes are the only liver cell type known to be transduced by AAV.[9] In animal models, stable transduction over a period of weeks to months following injection.[10] This is a shorter period of time for transduction than was observed after administration of AAV vector in muscle.[11] Using the liver-specific promoter, hepatocytes demonstrate restricted gene expression, which appears to provide an extra measure of safety.

This trial is enrolling adult men with severe hemophilia B. Patients may be HIV-positive and must have a negative history of inhibitor to factor IX. Patients who have evidence of active HCV infection (as demonstrated by presence of HCV-RNA by PCR assay) must have had a liver biopsy within the previous 2 years demonstrating minimal fibrosis.

To date, seven subjects have been enrolled. Preliminary results show that the subjects initially experienced no local or systemic toxicity.[12] Kay et al reported that one subject had a factor IX level of 13% weeks after treatment with the vector. The subject subsequently developed asymptomatic transaminitis with aspartate aminotransferase levels peaking at 500 U/L. With resolution of the transaminitis, the subject's factor IX level fell to 1%.[12]

Conclusions

The third-generation recombinant factor VIII products produced without added human- or animal-derived materials are in development and clinical trials. Two gene therapy trials, one in subjects with factor VIII and the other in subjects with factor IX deficiency, are currently under way, and results are expected in the near future.

With these new therapeutic options, the future for patients with hemophilia appears brighter than ever before. The current climate rightfully demands that clinical trials of new factor concentrates or gene therapy approaches be thoughtfully developed and implemented, with broad access to safety information. Preserving the health of research subjects as new therapies are developed is critical for maintaining a partnership between patients, their health care providers, and the affiliated industries.

References

1. National Hemophilia Foundation: MASAC Recommendations #135: MASAC recommendation concerning the treatment of hemophilia and other bleeding disorders (revised November 2002). Washington, DC, National Hemophilia Foundation. Available at: http://www.hemophilia.org/programs/masac/masac/masac135.htm. Accessed February 12, 2003
2. Tabor E: The epidemiology of virus transmission by plasma derivatives: Clinical studies verifying the lack of transmission of hepatitis B and C viruses and HIV type 1. Transfusion 39:1160-1168, 1999
3. Powell JS, Ragni MV, White GC, et al: Phase I trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. Thromb Haemost 86:OC2489, 2001 (suppl, abstr)
4. Roth DA, Tawa NE Jr, O'Brien JM, et al: Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. N Engl J Med 344:1735-1742, 2001
5. Manno CS, Glader B, Ragni MV, et al: A phase I trial of AAV-mediated muscle directed gene transfer for hemophilia B. Blood 96:801a, 2000 (suppl 1, abstr)
6. Manno CS, Chew AJ, Hutchinson S, et al: AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood 101:2963-2972, 2003
7. Reddy PS, Sakhuja K, Ganesh S, et al: Sustained human factor VIII expression in hemophilia A mice following systemic delivery of a gutless adenoviral vector. Mol Ther 5:63-73, 2002
8. GenStar: GenStar reports clinical results of the first patient to receive MAX-AD™ FVIII treatment for hemophilia A [press release]. July 11, 2001. Available at: http://www.genstar-rx.com/news/mainnews_frame.html. Accessed February 11, 2003
9. Miao CH, Nakai H, Thompson AR, et al: Nonrandom transduction of recombinant adeno-associated virus vectors in mouse hepatocytes in vivo: Cell cycling does not influence hepatocyte transduction. J Virol 74:3793-3803, 2000
10. Nakai H, Thomas CE, Storm TA, et al: A limited number of transducible hepatocytes restricts a wide-range linear vector dose response in recombinant adeno-associated virus-mediated liver transduction. J Virol 76:11343-11349, 2002
11. Qing K, Khuntirat B, Mah C, et al: Adeno-associated virus type 2-mediated gene transfer: Correlation of tyrosine phosphorylation of the cellular single-stranded D sequence-binding protein with transgene expression in human cells in vitro and murine tissues in vivo. J Virol 72:1593-1599, 1998
12. Kay MA, High KA, Glader B, et al: A phase I/II clinical trial for liver directed AAV-mediated gene transfer for severe hemophilia B. Blood 100:115a, 2002 (suppl 1, abstr)